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TITLE: Anti-Angiogenesis by a Novel VEGF-Intrakine Strategy for Breast Cancer Therapy

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Anti-Angiogenesis by a Novel VEGF-Intrakine Strategy for Breast Cancer Therapy

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Report contains color photos

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Angiogenesis plays a pivotal role in tumor growth and metastasis. VEGF, an endothelial specific mitogen and an angiogenesis inducer in vivo, is one of the most important tumor angiogenesis growth factor. KDR, a VEGF receptor, appears to be the major transducer of VEGF signals in endothelial cells. A tethered intracellular antibody ("intrabody") strategy has been used successfully for both phenotypic and functional knockouts of target molecules. In this study, we have targeted a KDR single chain antibody (scFv) p3S5 to the endoplasmic reticulum (ER) using a c-terminal endoplasmic retention signal (KDEL). We hypothesized that the tethered KDR intrabody would bind newly synthesized KDR and block its transport to the surface of endothelial cells, thereby inhibiting VEGF-induced proliferation. The tethered intrabody significantly reduced KDR expression (from 82.5 ± 12.5% to 27.9 ± 13.6%, P < 0.005) in successfully transfected cells. These results demonstrate the potential for using an intrabody strategy to block angiogenesis.
FOREWORD

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

L. Weeks 7/5/81

David Lane 7/3/81

PI - Signature Date
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(5). Introduction:

Tumor angiogenesis plays an important role in breast cancer growth and metastasis. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells after binding to its receptor (VEGF-R) on cell surfaces, and is a key factor in tumor angiogenesis. **The subject and scope of this research** involves generating various expression vectors for the VEGF-intrakine/intrabody and determining the effects of VEGF-intrakine/intrabody on the VEGF-R expression on endothelial cell surfaces, and evaluating the anti-angiogenesis and anti-tumor activity of this intrakine/intrabody. **The purpose of this study** is to inactivate VEGF-R in vascular endothelial cells by using this intrakine/intrabody strategy and thereby, preventing endothelial cell proliferation, which may lay the groundwork for the development of a novel approach for breast cancer therapy.

(6). Body:

Various VEGF-intrabody expression vectors have been generated by using series PCRs (Fig.1). The original plasmid that contains single chain antibody scFv p3S5 was obtained from Imclone Systems Incorporated (New York, NY). The scFv p3S5 cDNA was amplified by two PCRs with the sense primers (P1: 5’-GCTCCAGATGGGTCCTGTCGCAGGTGAACTGCAGGACTCA-3’ and P2: 5'-TTTGAATTGATGGAAGGATCTCCATGGAGGT-3'), and antisense primer (5’-TTTCTAGAGGATTTCACATGGATACGCGCCTTTCGATTTCCAAA-3’). An HA tag sequence (YPYDVPDYA) was linked to the scFv gene by a PCR reaction with the sense primer (P1 and P2), and antisense primer (5’-TTTCTAGAGGATTTCACATGGATACGCGCCTTTCGATTTCCAAA-3’). The scFv p3S5 gene tagged with HA was then linked with an ER retention signal (SEKDEL) by a PCR reaction with sense primer (P2) and antisense primer (5’-TTTCTAGAGGATTTCACATGGATACGCGCCTTTCGATTTCCAAA-3’). These DNA fragments were digested with BamHI and EcoRI, and cloned into the expression vector pIRES2-EGFP (Clontech). All of the constructs were identified by restriction enzyme digestion and confirmed by DNA sequencing (Sequencing Core Facility of Wake Forest University School of Medicine).

In order to determine the expression and intracellular localization of these intrabody vectors, these expression vectors were transfected into human umbilical vein endothelial cells using Lipofectamine reagent (Gibco BRL). The procedure followed was “Gibco transient or stable transfection of adherent cells protocol”. In brief, in a 35 mm tissue culture plate, 3 x 10^5 HUVECs were seeded and incubated at 37°C, 5% CO2 incubator overnight. 2 μg of plasmid DNA and 7 μl of lipofectamine reagent were diluted into 100ul OPTI-MEM I Reduced Serum Medium (Gibco BRL), respectively. The two solutions were mixed and incubated at room temperature for 30 minutes. Following incubation, 0.8ml OPTI-MEM I Reduced Serum Medium was added to the mixture. Then, the final solution was added to the 35 mm HUVECs plate and incubated at 37°C, 5% CO2 in an incubator for 5 hours. EGM-2 complete medium (0.8 ml) was added at the
last hour of incubation. Following incubation, fresh EGM-2 medium was added to the 
HUVECs and the cells were incubated in a 37°C, 5% CO2 incubator. In order to 
determine the localization of the modified p3S5, immunofluorescent staining was 
performed using an antibody to the HA tag. Figure 2 demonstrates that cytoplasmic ER 
staining pattern was observed in the p3S5-HAK transfected HUVECs. The expression of 
EGFP could be detected in transfected cells using fluorescence microscopy (Figure 2)...
Cells that did not express EGFP had no evidence of immunofluorescence with anti-HA 
(Fig 2), demonstrating specificity of the antibody and the lack of secretion of the 
intrabody with rebinding to neighboring cells. Cells transfected with the vector expressed 
EGFP, but did not exhibit immunofluorescence with anti-HA (Fig 2).

To examine the expression of modified p3S5-intrabody further, HUVECs were 
transfected with either pRES2-EGFP control or p3S5-HAK and 48 hours later, the cell 
lysates and concentrated culture medium were immunoblotted with an anti-HA antibody. 
A 30 kDa protein band corresponding to p3S5 was found exclusively in the cell lysate 
(Fig 3, lane 3) and not in the culture medium of p3S5-HAK transfected cells (Fig. 3, lane 
4). As expected, no immunoreactivity was found in control vector transfected cell lysates 
(Fig. 3, lane 1) or culture medium (Fig 3, lane 2).

To determine the effects of the modified p3S5-intrabody on KDR expression, HUVECs 
were transfected with pRES2-EGFP (control vector), p3S5-HA, or p3S5-HAK, and 48 
hour later, the cell surface expression of KDR was examined using flow cytometry 
(Figure 4). Two-color analysis was performed, with the results summarized in Table 1. 
Whereas 82.5 ± 12.5% of cells transfected with pRES2-EGFP expressed KDR, only 
27.9 ± 13.6% of cells transfected with p3S5-HAK expressed KDR (p<0.01). 
Transfection with the p3S5-HA vector without the KDEL tag was not effective in 
suppressing KDR expression with 78.6 ± 10.7% of the EGFP-expressing cells having 
detectable KDR (p>0.1). Even when all cells (GFP+ and GFP-) were included in the 
analysis, p3S5-HAK significantly reduced KDR expression (p<0.01).

To examine the effects of the modified p3S5-intrabody on HUVECs proliferation, 
HUVECs were transfected with p3S5-HAK or pRES2-EGFP control. After 48 hours in 
culture, they were sorted based on EGFP expression into transfected and non-transfected 
groups, then treated with VEGF₁₆₅ at 15 ng/ml for 30 hours. A [³H] thymidine 
icorporation assay was performed on these cells. The proliferation rate of the cells that 
had been transfected with p3S5-HAK was significantly lower than those from the same 
experiment that were not-transfected (p<0.005) (Fig. 5). Furthermore, there was no 
significant difference between non-transfected cells in the experimental group and the 
control vector groups. Thus, only the cells that expressed the p3S5-HAK construct had a 
significantly reduced response to VEGF₁₆₅.
(7). Key Research Accomplishments:

- Various intrabody expression vectors have been generated and expressed by HUVECs.
- Intrabody significantly decreased HUVECs surface expression of KDR, a VEGF receptor.
- Intrabody significantly inhibited HUVECs proliferation rate.

(8). Reportable Outcomes:

Presentation:
Journal Club, Cancer Biology Department of Wake Forest University School of Medicine: 03/2001.

Poster:

Paper submitted:

(9). Conclusions:

Various VEGF-intrabody expression vectors have been generated and successfully transfected into, and expressed by HUVECs. The intrabody has significantly decreased HUVECs surface expression of VEGF receptor, KDR. And they also significantly inhibited HUVECs proliferation rate in vitro. Further studies will be directed to investigate the mechanisms of the intrabody as well as some antitumor effects of intrabody in vivo.

(10). References:


(11). Appendices:

None.

(12). Final Reports:

One abstract has been accepted by the 4th Annual American Society of Gene Therapy Meeting (05/2001) and published on Molecular Therapy (05/2001).

One paper has been submitted to Blood (04/2001).

Principle investigator (Yurong Y. Wheeler) has received student payment from this award of $1,292.01/month for the last one year.
Fig. 2
Fig. 3
Table 1.

Analysis of flow cytometry assay of surface KDR expression on p3S5-HA, p3S5-HAK and control transfected HUVECs

<table>
<thead>
<tr>
<th>Transfected plasmid</th>
<th>Transfection percentage</th>
<th>% KDR positive</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>all cells</td>
<td>transfected cells</td>
</tr>
<tr>
<td>pIRES2-EGFP</td>
<td>5.1 ± 2.2</td>
<td>92.5 ± 3.7</td>
<td>(1) 82.5 ± 12.5</td>
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<tr>
<td>p3S5-HA</td>
<td>9.8 ± 6.9</td>
<td>94.1 ± 2.3</td>
<td>(2) 78.6 ± 10.7</td>
</tr>
<tr>
<td>p3S5-HAK</td>
<td>13.3 ± 3.1</td>
<td>85.3 ± 1.1</td>
<td>(3) 27.9 ± 13.6</td>
</tr>
</tbody>
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p-values (student t test):

(1) vs (3)  p < 0.01

(1) vs (2)  p > 0.1

(2) vs (3)  p < 0.01